REMARKS

Claims 1 and 3-17 are currently pending. No new matter has been added.

Applicants gratefully acknowledge the Examiner's withdrawal of the rejection of claims 1 and 3-17 under 35 U.S.C. §102(b) as being anticipated by U.S. Patent No. 5,844,003.

Rejection of Claims 1 and 3-17 under 35 U.S.C. §101

The rejection of claims 1 and 3-17 is maintained under 35 U.S.C. §101 because, as asserted in the Office Action mailed on November 15, 2006, "the claimed invention is not supported by either a specific asserted utility or a well established utility." Specifically, the Examiner asserts that the phrase "rescuing damaged nerve cells" is non-specific.

Applicants respectfully traverse. Applicants respectfully submit that a skilled artisan at the time of the filing of the application would have reasonably understood that rescuing damaged nerve cells would be a useful process for treating disorders in a patient, as evidenced by Tessler, A. "Interspinal Transplants" *Ann. Neurol.* (1991) 29:115-123 and Strömberg, I. *et al.* "Rescue of Basal Forebrain Cholinergic Neurons after Implantation of Genetically Modified Cells Producing Recombinant NGF" *Journal of Neurological Research*(1990) 25:405-411 (attached herewith as Appendices A and B, respectively).

The Tessler reference discloses "strategies that have been proposed for using intraspinal transplants to reverse the deficits due to spinal cord damage" in subjects. One strategy disclosed by Tessler involves the transplant "of fetal spinal cord in an attempt to replace damaged populations of neurons and encourage the restoration of connections between neurons" and one "mechanism provided by transplants might be ***the rescue of axotomized neurons*** in the spinal cord." (see page 117, left column, last paragraph). Moreover, Tessler also discloses that transplants of embryonic spinal cord that contributes to recovery of behavioral function was first described in newborn rat hosts, where ***transplants rescued axotomized host rubrospinal neurons that otherwise would have died*** and that "***[a]xotomized neurons of Clarke's nucleus can also be rescued by embryonic transplants*** in newborns, and a rescue of similar magnitude has now been reported when the same population of neurons is axotomized in adult animals."

The Strömberg *et al.* reference also discloses the rescue of nerve cells and specifically, the ***rescue of basal forebrain cholinergic neurons*** by implantation of genetically modified cells producing recombinant nerve growth factor in order to prevent degeneration and death of the neurons. In particular, Strömberg *et al.* discloses that

> [g]rafts of the genetically modified NGF producing cells ***rescued axotomized basal forebrain cholinergic neurons*** and significantly reduced cholinergic cell death in the medial septum as compared with rats treated with grafts of the parental 3T3 cells. Grafted fibroblast cells were detected, and rescue effects were noted up to 6 weeks after grafting. (*see* Abstract)

The Strömberg *et al.* reference further suggest that rescuing damaged nerve cells may have therapeutic applications in the treatment of senile dementia of Alzheimer's disease and Parkinson's disease.

Accordingly, a skilled artisan at the time the invention was made would realize that rescuing damaged nerve cells would have implications in the treatment of neurological disorders and therefore would be useful. Therefore, Applicants respectfully request reconsideration and withdrawal of this rejection.

Rejection of Claims 1 and 3-17 under 35 U.S.C. 112, first paragraph

The rejection of claims 1 and 3-17 under 35 U.S.C. §112, second paragraph, as allegedly failing to comply with the enablement requirement is maintained. Specifically, the Examiner asserts in the Office Action mailed on November 16, 2006, that "one of ordinary skill in the art would be burdened with undue experimentation to determine all neurodegenerative and neuromuscular disorders which can be treated with the claimed deprenyl compounds."

Applicants respectfully traverse. Applicants respectfully submit that the test of enablement is whether one reasonably skilled in the art could make or use the invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (Fed. Cir. 1988). See also *United States v. Telectronics, Inc.*, 857 F.2d 778, 785, 8 USPQ2d 1217, 1223 (Fed. Cir. 1988). There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to: (A) The breadth of the claims; (B) The nature of the invention; (C) The state of the prior art; (D) The level of one of ordinary skill; (E) The level of predictability in the art; (F) The amount of direction provided by the inventor; (G) The existence of working examples; and (H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure. The determination that "undue experimentation" would have been needed to make and use the claimed invention is not a single, simple factual

determination. Rather, it is a conclusion reached by weighing all the above noted factual considerations. *In re Wands,* 858 F.2d at 737, 8 USPQ2d at 1404.

Nature of the Claims

The currently pending claims are directed to methods of rescuing damaged nerve cells in a subject by administering to a subject with damaged nerve cells a deprenyl compound of Formula I provided that the deprenyl compound is not deprenyl, pargyline, AGN-1133 or AGN-1135.

Breadth of the Claims

The Examiner asserts that "[t]he claims are very broad and encompass a composition for treating all disorders associated with damaged nerve cells."

Applicants disagree with this characterization of the claims. Applicants respectfully submit that the claimed invention is directed to ***methods of rescuing damaged nerve cells, not methods of treating neurodegenerative or neuromuscular disorders.***

State of the Prior Art

With respect to the state of the prior art, the Examiner asserts in the previous Office Action mailed on November 16, 2006, that "[t]he prior art does not recognize that all neurodegenerative neuromuscular disorders are the same in nature and can be treated in the same manner." As stated above, Applicants respectfully submit that the claimed invention is directed to methods of rescuing damaged nerve cells, not methods of treating neurodegenerative or neuromuscular disorders.

Moreover, as evidenced by the Tessler and Strömberg *et al.* references described immediately above, at the time the invention was made, ***the prior art not only recognized the usefulness of rescuing damaged nerve cells, but also had developed methods for determining whether nerve cells had been rescued.***

Level of Skill in the Art

As the Examiner acknowledges, the level of skill of an artisan practicing the present invention is high.

Level of Predictability if the Art

The Examiner asserts that "[t]he claims are very broad and encompass a composition for treating all disorders associated with damaged nerve cells." Applicants respectfully disagree.

Specifically, the claims are directed ***to rescuing damaged nerve cells***, not treating all disorders associated with damaged nerve cells.

Moreover, Applicants respectfully submit, as evidenced by Tessler and Strömberg *et al.*, that at the time the invention was made, experimental methods for determining if nerve cells had been rescued were established. Accordingly, a skilled artisan would have been able to use the specification as filed in combination with the general knowledge in the art to make and use the claimed invention. Moreover, all that is necessary is that one skilled in the art be able to practice the claimed invention, given the level of knowledge and skill in the art. (*see* MPEP 2164.08).

The Amount of Direction Provided by Applicants and Presence of Working Examples

The Examiner asserts that "Applicant's specification provides no guidance as how the treatment of disorders associated with damaged nerve cells is accomplished" and that "Applicants specification does not set forth any examples to demonstrate that the claimed compounds are capable of treating any disorders associated with damaged nerve cells." The Examiner also asserts that no correlation has been established between such [protective effect of deprenyl] and treating neurodegenerative, neuromuscular disorders." The Examiner further asserts that "one of ordinary skill in the art would be burdened with undue experimentation to determine all neurodegenerative and neuromuscular disorders which can be treated with the claimed deprenyl compounds."

Applicants respectfully traverse. Compliance with the enablement requirement of 35 U.S.C. §112, first paragraph, does not turn on whether an example is disclosed. An applicant need not have actually reduced the invention to practice prior to filing. The specification need not contain an example if the invention is otherwise disclosed in such manner that one skilled in the art will be able to practice it without an undue amount of experimentation. *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970). (*see* MPEP 2164.02).

With regard to the "correlation," Applicants respectfully submit that "correlation" refers to the relationship between *in vitro* or *in vivo* animal model assays and a disclosed or a claimed method of use. An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. Based upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence. (*see* MPEP 2164.02) A rigorous or an invariable exact correlation is not required, as stated in *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985).

The Examiner noted in the previous Office Action, "[t]he examples in Applicant's specification are drawn to the protective effect of deprenyl on rescuing nerve cells *in vitro*." As the claimed methods are directed to ***rescuing damaged nerve cells***, a skilled artisan would have appreciated that the *in vitro* protective effects of deprenyl might correlate to a protective effect *in vivo*.

Upon weighing all of the above-listed factors, Applicants respectfully submit that a skilled artisan would have been able to use the examples disclosed in the specification, in combination with the general knowledge in the art, to make and use the claimed invention of rescuing damaged nerve cells with a deprenyl compound of Formula I.

Based at least on the foregoing, Applicants respectfully request reconsideration and withdrawal of the rejection of claims 1 and 3-17 under 35 U.S.C. §112, first paragraph.

Rejection of Claims 1 and 3-17 Under the Judicially Created Doctrine of Obviousness-type Double Patenting

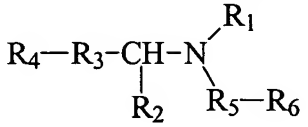
The rejection of claims 1 and 3-17 on the grounds of nonstatutory obviousness-type double patenting as allegedly being unpatentable over claims 1-14, 19 and 21 of U.S. Patent No. 5,844,003 (the '003 patent) is maintained. Specifically, the Examiner asserts in the Office Action mailed on November 16, 2006, that "[t]he claims of the instant application are drawn to a method for rescuing damaged nerve cells in a patient using a deprenyl compound with the exclusion of certain deprenyl compounds" and that claims 1-14, 19 and 21 of the '003 patent "are directed to a method of rescuing damaged nerve cells in a patient using a specific deprenyl compounds." The Examiner concludes that "[s]uch compounds are within the scope of the claimed compounds."

Applicants respectfully submit that, while in no way admitting that the present claims are obvious over claims 1-14, 19 and 21 of U.S. Patent No. 5,844,003, upon allowance of the present claims, Applicants will consider submitting a terminal disclaimer in compliance with 37 C.F.R. 1.321(b) and (c), if appropriate, which will obviate the rejection.

Rejection of Claims 1 and 3-17 under 35 U.S.C. §102(b)

Claims 1 and 3-17 are rejected under 35 U.S.C. §102(b) as allegedly being anticipated by Parkinson, S.G. *Arch. Neuron* (1989) 46:1052-1060. Specifically, the Examiner asserts that Parkinson "teaches the use of deprenyl compounds of the treatment of Parkinson disease" and that "[s]uch use would inherently rescue damaged nerve cells."

Applicants respectfully traverse. The pending claims are directed to methods of rescuing damaged nerve cells by administering to a patient having damaged nerve cells an amount of a deprenyl compound, wherein the deprenyl compound is represented by the structure of Formula I:



with the proviso that the ***deprenyl compound is not selected from the group consisting of deprenyl,*** pargyline, AGN-1133, or AGN1135.

In contrast, Parkinson describes "a placebo-controlled clinical trial designed to test the hypothesis that ***long-term treatment of patients with early Parkinson's disease with deprenyl 10 mg/d*** and/or tocopherol (vitamin E) 2000 IU/D will extend the time until disability requires therapy with Levadopa." Parkinson further describes that the patients were given (1) active deprenyl; (2) active tocopherol; (3) active deprenyl and tocopherol; or (4) placebo treatments.

However, Parkinson fails to teach or suggest rescuing damaged nerve cells with compounds of Formula I. Moreover, ***since claim 1 expressly excludes deprenyl,*** Applicants respectfully submit that Parkinson does not teach or suggest every element of the claims, and therefore fails to anticipate the claimed invention. Accordingly, Applicants respectfully request reconsideration and withdrawal of this rejection of claim 1 and 3-17 under 35 U.S.C. §102(b).

SUMMARY

In view of the foregoing, it is respectfully submitted that this application is in condition for allowance. If there are any remaining issues or the Examiner believes that a telephone conversation with Applicants' Attorney would be helpful in expediting prosecution of this application, the Examiner is invited to call the undersigned at (617) 227-7400.

Dated: February 11, 2008

Respectfully submitted,

By _____

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Appendix A

Intraspinal Transplants

Alan Tessler, MD

Transplants of embryonic central nervous system tissue have long been used to study axon growth during development and regeneration, and more recently to promote recovery in models of human diseases. Transplants of embryonic substantia nigra correct some of the deficits found in experimental Parkinson's disease, for example, by mechanisms that are thought to include release of neurotransmitter and reinnervation of host targets, as well as by stimulating growth of host axons. Similar mechanisms appear to allow intraspinal transplants of embryonic brainstem to reverse locomotor and autonomic deficits due to experimental spinal cord injuries. Embryonic spinal cord transplants offer an additional strategy for correcting the deficits of spinal cord injury because, by replacing damaged populations of neurons, they may mediate the restoration of connections between host neurons. We have found that spinal cord transplants permit regrowth of adult host axons resulting in reconstitution of synaptic complexes within the transplant that in many respects resemble normal synapses. Transplants of fetal spinal cord may also contribute to behavioral recovery by rescuing axotomized host neurons that otherwise would have died. Electrophysiological and behavioral investigations of functional recovery after intraspinal transplantation are preliminary, and the role of transplants in the treatment of human spinal cord injury is uncertain. Transplants are contributing to our understanding of the mechanisms of recovery, however, and are likely to play a role in the development of rational treatments.

Tessler A. Intraspinal transplants. Ann Neurol 1991;29:115–123

Transplants of embryonic central nervous system tissue have served for nearly a century in experiments designed to clarify the mechanisms that contribute to axon outgrowth and regeneration (reviewed in [1]). Throughout the past 20 years, transplants have been used in attempts to produce physiological or behavioral improvement in laboratory models of human diseases and to study the mechanisms that explain recovery of function (reviewed in [2–4]). Transplants remained primarily of theoretical interest for clinicians, however, until reports that autografts of adrenal medulla into the caudate nucleus produced modest [5] or dramatic [6] improvement in the motor behavior of patients with Parkinson's disease. More than 250 patients with Parkinson's disease have received adrenal medulla autografts [7], and additional patients have received transplants of human fetal substantia nigra [8–10]. The benefits of these procedures continue to be debated [11–17], and the mechanisms by which transplants elicit their effects continue to be investigated.

Transplants have also been reported to produce improvement in experimental models of Huntington's disease [2], Alzheimer's disease [18], and spinal cord injury [19–25]. Intraspinal transplantation would seem to be far from clinical application because electrophysiological and behavioral investigations of functional recovery are still preliminary. Regions of embryonic brain [26–35] as well as whole pieces or suspensions prepared from embryonic spinal cord survive transplantation into the spinal cord of adult and newborn host rats [36–38]. Intraspinal transplantations have also been successful in cat [21, 39, 40] and monkey. The extent to which connections form between transplant and host is beginning to be studied with morphological techniques.

In the present review, the strategies that have been proposed for using intraspinal transplants to reverse the deficits due to spinal cord damage will be considered, and the progress that has been made will be outlined. Because the effects of transplants have been most thoroughly studied in the basal ganglia in relation to Parkinson's disease, I first consider the mechanisms by which transplants may reverse the behavioral deficits in experimental models of this disorder.

Transplants for Experimental Parkinson's Disease

Transplantation strategies for the treatment of Parkinson's disease were built on a foundation of reproducible and quantifiable experimental models (reviewed in [41]). The first of these was a rodent model in which the substantia nigra corpus striatum projection was destroyed by the unilateral or bilateral stereotactic injection of the dopaminergic neurotoxin 6-hydroxydopa-

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Received Jun 21, 1990, and in revised form Aug 27. Accepted for publication Sep 4, 1990.

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mine (6-OHDA). When fetal substantia nigra was placed into the lateral ventricle adjacent to the corpus striatum that had been denervated by 6-OHDA, transplanted neurons survived and formed a dense dopaminergic innervation within the grafts, but few processes extended into the host corpus striatum [42]. In spite of the limited reinnervation, the transplants improved some aspects of motor behavior. This result suggested that reinnervation was not necessary for recovery and that release of dopamine into the cerebrospinal fluid and in the vicinity of the target neurons sufficed. If fetal substantia nigra is transplanted into the parenchyma of the corpus striatum denervated by 6-OHDA injection rather than into the ventricle, then the transplants partially restore the damaged neural circuits and additional behaviors recover [43–47]. At least some of the recovery is related to reinnervation of the host striatum (reviewed in [41, 48]). Other behavioral deficits induced by unilateral dopamine deafferentation, however, remain uncorrected by the transplants. Incomplete behavioral recovery has been attributed to incomplete restoration of the damaged regulatory circuitry [47].

Another mechanism by which transplants may compensate for behavioral deficits has been demonstrated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of experimental Parkinson's disease [49, 50]. When adrenal medullary cells are transplanted into the striatum of mice intoxicated with MPTP, the mice recover in spite of a very limited survival of grafted cells [51]. The expression of tyrosine hydroxylase, a marker for dopaminergic neurons, increases within the caudate ipsilateral to the site of MPTP injection, and derives from axons of the host dopaminergic projection rather than from the few surviving transplanted neurons [51]. Increased enzyme expression indicates regeneration of damaged host axons or collateral sprouting of undamaged host axons and suggests that the transplant or the damage associated with the transplant procedure [52] exerts a trophic effect on the host neurons that contributes to behavioral recovery but is independent of graft survival.

In summary, this experience with experimental Parkinson's disease indicates that some types of motor function can be improved by transplants that deliver neurotransmitters, trophic factors, or both, to the striatum even if the transplants fail to reconstitute the damaged neural circuits in all their detail. Because restitution of the damaged circuits is incomplete, however, the motor deficits are only partially compensated, and behavioral recovery is also incomplete.

Intraspinal Transplants

As elsewhere in the central nervous system, intraspinal grafts of peripheral nerve segments support axon growth [53, 54]. These experiments have contributed to the current view that many neurons once thought incapable of growth can elongate if provided with a suitable glial environment (reviewed in [55]). Although central nervous system axons can grow within sciatic nerve grafts for distances that exceed their normal length [56] and establish synaptic connections with neurons in the host parenchyma [57, 58], growth into the central nervous system beyond the graft is limited to 1 to 2 mm [55]. Two other strategies for using transplants to treat experimental spinal cord injury have therefore received considerable attention.

Intraspinal Transplants of Supraspinal Neurons

One strategy is based on the idea that transplantation of brainstem monoaminergic neurons important for regulating the activity of spinal neurons should mediate recovery even without the reconstitution of damaged neural circuits ([30] and reviewed in [35]). Both locomotor and autonomic functions might benefit. It is known, for example, that the intravenous administration of dopamine or the alpha$_2$-adrenergic agonist clonidine to cats with acute spinal cord transection can elicit stepping movements that permit walking on a treadmill [59]. Untreated, such cats are completely paraplegic after transection. These agents are thought to act directly on lumbar spinal cord segments to activate the circuitry for locomotion that is intrinsic to the spinal cord [60] but nonfunctional in the acute stage after transection. It is also known that serotoninergic neurons whose perikarya are in the brainstem raphe nuclei are important in the supraspinal control of spinal reflexes that mediate penile erection and ejaculation, and that administration of a serotonin receptor agonist to spinalized rats can induce ejaculation [61].

One approach to the treatment of behavioral deficits after experimental spinal cord injury has been to transplant supraspinal monoaminergic neurons into the caudal portion of the spinal cord isolated by transection. Both catecholaminergic neurons important for locomotion and serotoninergic neurons important for autonomic function have been used [62]. Embryonic noradrenergic neurons taken from locus ceruleus [31–35] and serotoninergic neurons taken from the mesencephalic or medullary raphe regions [23, 24, 63, 64] extend processes up to 1 to 2 cm in length into the host spinal cord. These transplants restore levels of neurotransmitters that have been depleted by the use of neurotoxins [34] or by spinal cord transection [23, 24]. Axons of transplanted serotoninergic neurons innervate the regions of spinal cord that receive 5-hydroxytryptamine (5-HT) innervation normally, including laminae I and II of the dorsal horn, the motoneuron area in lamina IX of the ventral horn, and the intermediolateral column [23, 24]. The transplanted serotoninergic axons establish axodendritic synapses on host motoneurons and axodendritic and axosomatic

synapses on neurons of the host intermediolateral column that resemble those formed by serotoninergic neurons in normal spinal cord [23, 24]. Additionally, reflex ejaculation that is abolished in rats with spinal cord transection recovers in rats that receive serotoninergic transplants of embryonic raphe neurons, but only rarely in rats that undergo transection alone or transection with transplantation of nonserotoninergic neurons [23, 24]. This result suggests that behavioral recovery is related to the recovery of serotoninergic innervation.

Noradrenergic axons originating in locus ceruleus transplanted into spinal cord also extend into the intermediate and ventral regions of the host caudal gray matter [34] to which locus ceruleus axons project in normal spinal cord. These axons are thought to contribute to the recovery of hindlimb flexion reflexes in rats whose spinal cord catecholamines have been depleted by an intracisternal injection of 6-OHDA [20] and to the recovery of reflex stepping activity in rats with spinal cord transections [25]. Whether transplanted noradrenergic axons establish synapses with host neurons has not been studied, and therefore, the anatomical basis for the locomotor recovery mediated by the transplants is uncertain. Like intraparenchymal transplants of substantia nigra in models of Parkinson's disease, these embryonic transplants of brainstem neurons appear to reinnervate a portion of their normal targets and contribute to behavioral recovery by releasing neurotransmitters onto their normal targets or in their vicinity. The release of these neurotransmitters would then mimic the modulatory activity of descending noradrenergic systems in normal spinal cord and suffice to activate the intrinsic spinal pattern generators for locomotion [34, 60]. As in the corpus striatum deafferented by 6-OHDA, the transplants are successful although they have been placed into abnormal locations and can only incompletely restore the interrupted neural circuits.

Transplants of Fetal Spinal Cord

The recovery of other functions lost after spinal cord injury such as discriminatory sensation or fine motor control is likely to require more faithful reconstruction of normal circuitry than is possible by using transplants of supraspinal neurons. A second strategy has therefore been to use transplants of fetal spinal cord in an attempt to replace damaged populations of neurons and encourage the restoration of connections between neurons [36, 65]. According to this rationale, the transplant would act as a bridge across damaged tissue either by allowing injured axons to grow directly into intact spinal segments or by permitting the establishment of relays within the graft (Fig 1). Another mechanism provided by transplants might be the rescue of axotomized neurons in the spinal cord. Although the spinal cord has been considered a technically challenging site in

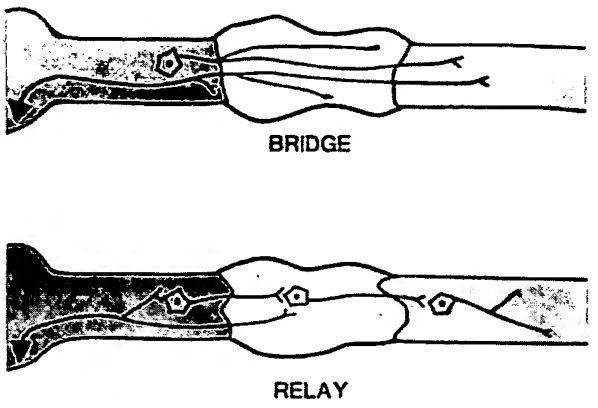
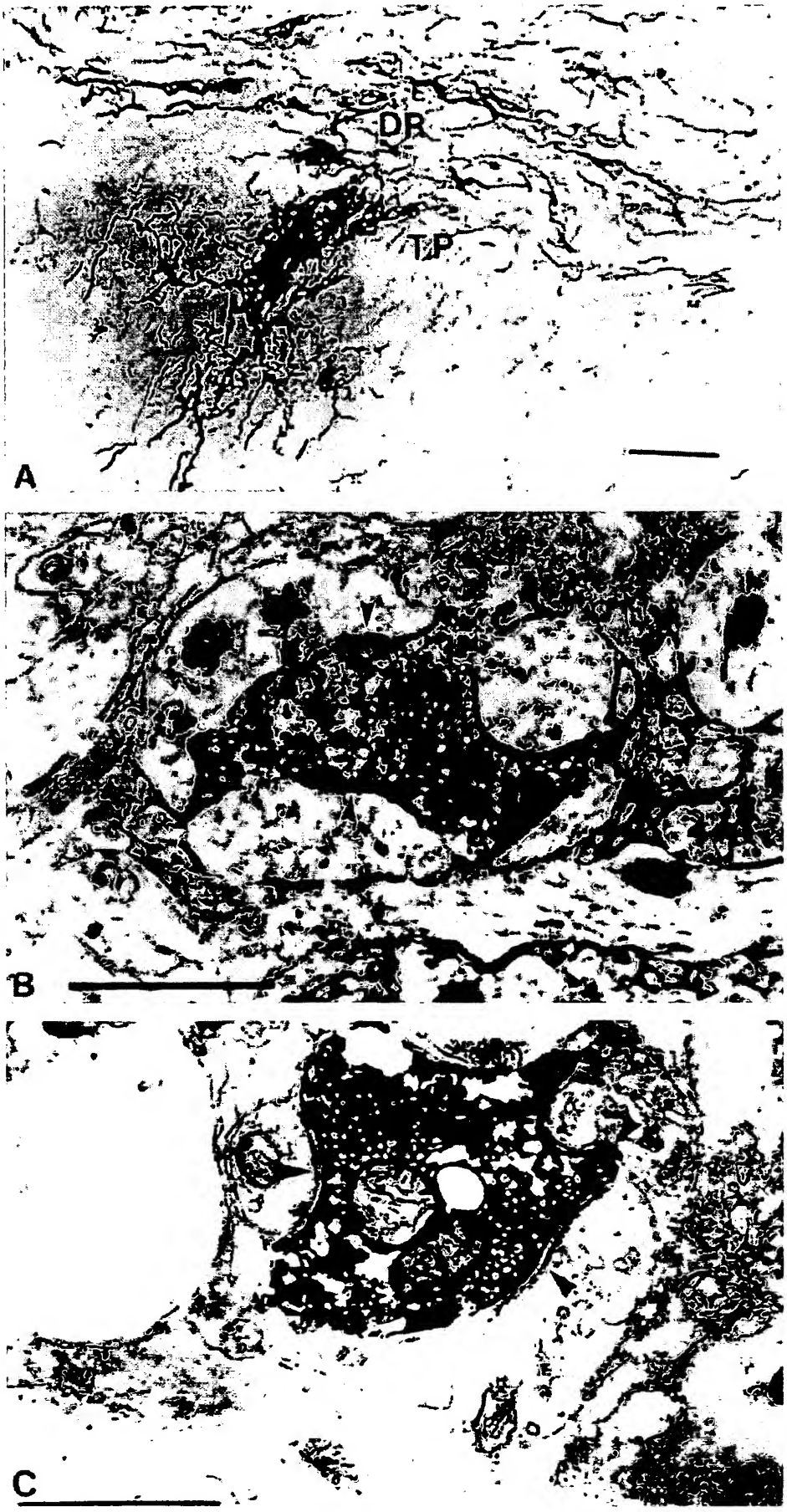


Fig 1. A diagram illustrating two ways in which a transplant (shaded area) at a region of spinal cord injury might promote functional recovery. Host brain and spinal cord rostral to the transplant are to the left and host caudal spinal cord is to the right. (Bridge) Injured axons originating in host neurons rostral to the transplant grow directly through the transplant into caudal host spinal cord. (Relay) Injured axons originating in host neurons rostral to the transplant grow into the transplant and synapse with donor neurons whose axons grow into caudal host spinal cord. (Courtesy of Dr Barbara Bregman.)

which to obtain survival of embryonic spinal cord transplants, with present methods 80 to 90% of transplants survive grafting into the acutely injured adult or newborn spinal cord [28, 36] or into the chronically injured adult spinal cord [37]. Transplant survival is now possible also in the site of complete spinal cord transection [21, 66, 67].

Several morphological features of the transplants encourage the expectation that they might replace damaged populations of neurons and with them their normal connections. First, although transplants lack the characteristic butterfly appearance of normal spinal cord gray matter, they do contain differentiated areas that resemble substantia geltatinosa based on several morphological criteria [68]. Second, areas of apposition between transplant and host develop, particularly between transplant and host spinal cord gray matter, in which the interface is free of astrocytic scarring and processes pass between transplant and host [36]. It has, in fact, been suggested both that embryonic transplants reduce the astrocytic scarring that accompanies acute spinal cord injury and may impede regeneration [69, 70] and that they can reduce an already established astrocytic scar [37].

One additional way in which transplants of embryonic spinal cord may contribute to recovery of behavioral function was first described in newborn rat hosts, where transplants rescue axotomized host rubrospinal neurons that otherwise would have died [71]. Permanent rescue is target specific because only transplants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of fetal spinal cord support survival at long survival periods; both target and nontarget transplants support short-term survival [72]. Axotomized neurons of Clarke's nucleus can also be rescued by embryonic transplants in newborns, and a rescue of similar magnitude has now been reported when the same population of neurons is axotomized in adult animals [73].

In adults, development of projections from host into transplant and from transplant into host is modest. Host axons that have grown into the transplants do not traverse their full extent and do not reenter host spinal cord [36]. Morphological tracing methods have shown that donor neurons project for distances of 5 to 7 mm into host spinal cord and that host neurons in adjacent spinal segments project from 3 to 5 mm into the transplants [36]. Additionally, serotoninergic axons from brainstem raphe nuclei [36], axons from corticospinal neurons [74, 75], and primary afferent axons originating in host dorsal root ganglion neurons [67, 76] regenerate into transplants although they are unable to regenerate into adult spinal cord in the absence of a transplant (Fig 2). The terminals of regenerated dorsal root axons establish synapses within transplants of embryonic spinal cord, and these synapses resemble those formed by primary afferent axons in the dorsal horn of normal animals [38] (Fig 3). Differences were also observed, however, particularly an increased percentage of axo-axonic synapses suggesting that regenerated dorsal root axon terminals had formed connections with each other. Nevertheless, the establishment of synapses that are morphologically normal by an identified set of host neurons afferent to the transplants encourages the idea that neurons joined by synapses within the transplants may enable the transplants to act as relays across regions of damaged spinal cord. Donor neurons within transplants have in fact been shown to send axons into the host sciatic nerve [76] as well as into peripheral nerve grafts introduced into the transplants [77, 78]. Whether these axons reach and innervate host muscles is unknown. It is, however, at least possible that transplants of embryonic spinal cord not only can support or enhance the regeneration of adult axons otherwise unable to grow but also that they can contribute to the reestablishment of a segmental motor reflex arc.

In newborn rat hosts, spinal cord transplants act as bridges that encourage or permit the growth of supraspinal neurons into segments of spinal cord well below the level of injury. Serotoninergic axons originating in neurons of the brainstem raphe [79] and axons of corticospinal neurons [75] grow across transplants placed in the lesioned thoracic spinal cord of newborn rats and terminate in their normal target areas as far caudal as the lower lumbar segments of host spinal cord. Because these systems of neurons are among the descending pathways that contribute to the control of locomotion, the possibility has been tested that transplants alter the development or enhance the recovery of motor function after spinal cord injury in newborn rats with partial spinal cord lesions [22]. When studied with a battery of tests of locomotor function, newborn rats with transplants of embryonic spinal cord performed better than littermates with thoracic spinal cord injuries alone. For example, when tested 8 to 12 weeks postoperatively, rats with transplants crossed a mesh runway more quickly and made fewer errors in foot placement than the lesion-only group of rats. They also recovered more quickly from their errors. These results are consistent with the idea that the axons that grow into host spinal cord caudal to the thoracic spinal cord injury contribute to the improved performance. Because the rats in this study sustained an incomplete injury rather than complete spinal cord transection, however, other explanations cannot be excluded. One possibility is that the transplants altered the response to injury of the remaining spinal cord and slowed or decreased degeneration, thus providing a greater amount of healthy tissue through which developing or regenerating axons might grow. Corticospinal axons are known to grow through undamaged areas of spinal cord adjacent to an incomplete spinal cord injury in newborns [80–82], and transplants of cultured dorsal root ganglia neurons and Schwann cells have been shown to enhance this growth [83]. Although corticospinal axons grow more robustly through transplants than through the remaining portions of spinal cord [75], supraspinal axons that have grown through host spinal cord as well as those that have grown through the transplant may contribute to the recovery.

Locomotor function is also being studied in cats that have received embryonic spinal cord transplants into the site of spinal cord transection on the day after birth. These preliminary studies on cats with transections complement those performed in rats with hemisections because a more detailed analysis of locomotor function is possible in cats. Additionally, the anatomical pathways that explain performance on different tests of mo-

Fig 2. Light (A) and electron (B,C) micrographs of embryonic spinal cord transplants. (A) Sagittal section 1 month after transplantation stained with an immunocytochemical method for demonstrating calcitonin gene-related peptide (CGRP). CGRP originates in many dorsal root ganglion neurons and serves as a marker for host dorsal roots (DR) that have regenerated into the transplant (TP) and arborized there [29, 38, 86]. Bar = 100 μm. (B) CGRP-labeled complex terminal in a transplant makes contacts (arrowheads) *with two dendritic profiles. Bar = 1 μm. (C) Host dorsal roots labeled with horseradish peroxidase (HRP). Complex terminal that has been filled with HRP makes synaptic contacts* (arrowheads) *in a transplant with three dendritic profiles. Bar = 1 μm.*

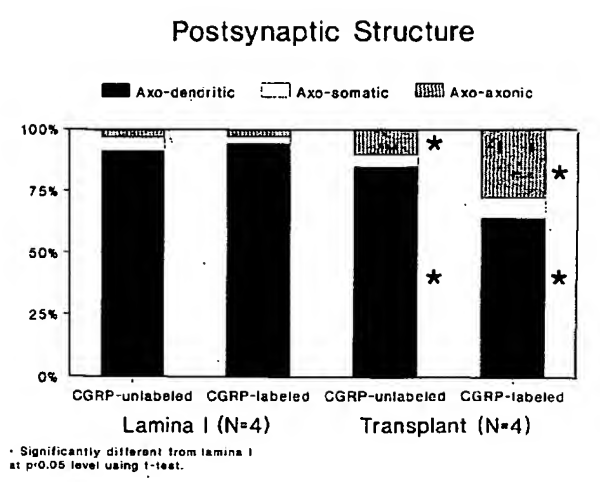

Fig 3. Comparison of synaptic contacts found in embryonic spinal cord transplants and lamina I of normal spinal cord. Calcitonin gene-related peptide (CGRP)-labeled synaptic terminals are derived from dorsal roots; the origin of unlabeled synaptic terminals is undetermined. Compared with lamina I, both CGRP-labeled and CGRP-unlabeled terminals in transplants make significantly greater numbers of axo-axonic synapses and significantly smaller numbers of axo-dendritic synapses. Axo-dendritic synapses predominate in transplants, however, as in lamina I. For details of the stereological analysis see [38].

tor function are better defined in cats than rats. Previous studies in normal [84] and cats with spinal cord transection [85, 86, 87] have demonstrated three types of locomotion that are available for analysis and that are controlled by different classes of spinal pathways [88]. For example, after resolution of the period of reflex depression known as spinal shock, hindlimb locomotion in response to a moving treadmill (bipedal reflex locomotion) returns in adult cats with spinal cord transection (spinal cats) because this type of locomotion requires only that the spinal pattern generators for each hindlimb and the connections between them remain intact [86]. Reflex locomotion on a treadmill that requires coordination between the forelimbs and hindlimbs (quadrupedal reflex locomotion) does not recover after thoracic transection because it depends on propriospinal connections between forelimb and hindlimb pattern generators in the cervical and lumbar spinal cord, and these are interrupted by the transection. Conditioned (voluntary) overground locomotion for a food reward will also not recover after transection either in adult or newborn cats because it depends not only on intact segmental and intersegmental connections but also on the presence of pathways that originate in the brain. In cats with thoracic spinal cord transections that have received a transplant on the day after birth, the presence of quadrupedal reflex locomotion will, therefore, suggest that the transplant has encouraged the growth of propriospinal connections across the site of transection, and the presence of conditioned overground locomotion will suggest the growth of axons with cell bodies in the brain.

Locomotor function has been examined in two cats that received transplants of E-26 spinal cord into T-12 transections on the day after birth [21]. These cats were compared with normal cats [89] and with a previously reported group of cats with transections as newborns but which did not receive transplants (spinal cats) [86]. By 3 weeks of age, the transected cats with transplants begin to differ from the spinal cats and the differences are maintained throughout the period of study. All groups of cats develop bipedal stepping, but unlike spinal cats, those with transplants also develop both full weight-supported quadrupedal stepping on a treadmill and overground locomotion. During overground locomotion, cats with transplants develop patterns of limb movement that suggest coordination between hindlimbs and forelimbs. The coordination is only sometimes similar to that seen in normal adult animals and overground locomotion is abnormal. For example, the postural stability of the hindquarters is impaired, the step cycle is prolonged, and the normal 1:1 pairing of forelimb and hindlimb step cycles is inconsistent. When the cats were studied histologically 2.5 and 8 months after transplantation, transplants filled the entire lesion cavity and were in continuity with host spinal cord in some places. Both transplants developed areas of gray matter and ependymal elements, and contained healthy appearing neurons and glial cells. These preliminary results indicate that transplants enhance the development or recovery of locomotor function in newborns with spinal cord transection and suggest that this effect is mediated by the axons of propriospinal or supraspinal neurons, or both, that enter the transplants. Additional experiments using anatomical tracing methods are necessary to establish this and to determine whether these axons traverse the transplant or form multisynaptic relays within the transplant. If the locomotor behavior in fact depends on supraspinal input, then transplants into newborn spinal cord will have assisted in the development or recovery of complex behavior that requires the reconstitution of supraspinal input and control of these supraspinal neurons by the host. Like the transplants for experimental Parkinson's disease, these transplants restore some of the lost functions but not the complete array.

Greater recovery may be obtained by combining transplants with additional agents that encourage axon growth. When exposed to the joint influences of a fetal spinal cord transplant and a prosthesis containing nerve growth factor (NGF), for example, cut dorsal root axons grow into and past the transplant into the ventral horn of host spinal cord [90]. Without added NGF, dorsal roots grow into the grafts but not through them

into host spinal cord [76]. NGF is the best studied neurotrophic factor and is known to elicit axon outgrowth in vitro [91]. In addition to growth factors, other agents are becoming available that, together with a transplant, might promote growth past an area of damage and into undamaged spinal cord. Increasing experimental evidence now emphasizes the importance of inhibitory influences in directing and limiting axon growth [92–95], and the molecules responsible are beginning to be identified. Caroni and Schwab [96, 97], for example, have demonstrated that peptides present in central nervous system myelin inhibit axon extension [96] and that this inhibitory activity can be blocked with a monoclonal antibody against the peptides [97]. Application of these antibodies to young rats whose corticospinal axons have been transected in the thoracic spinal cord is associated with sprouting of these axons at the lesion site and limited growth into the caudal spinal cord [98]. Scarring at the lesion site may obstruct the growth of many of these sprouted axons [70, 99]. Because transplants are reported to reduce gliosis and support axon extension across an area of injury, growth and associated recovery might be greater if, in addition to reducing inhibitory influences, the lesion site were bridged with a transplant.

Conclusion

Intraspinal transplants have clarified the conditions necessary for axon growth and regeneration, and have provided strategies that can contribute to behavioral recovery in experimental models of disease. Many issues remain to be resolved in the laboratory before the therapeutic potential of transplants can be evaluated. That injured central nervous system neurons can survive and grow in an appropriate environment, however, increases the likelihood that rational therapies to promote recovery will be forthcoming and makes more urgent the basic studies on which the development of these therapies will depend. Although it is not yet clear whether transplants will be part of the treatment of human spinal cord disease, they will at least have a role to play in developing treatments.

Research supported by the Research Service of the Veterans Administration, United States Army Medical Research and Development Command Grant 5190002, and National Institutes of Health Grant NS-24707.

I am grateful to M. E. Goldberger, B. T. Himes, and M. M. Murray for their critical reading of the manuscript, to B. T. Himes for his excellent technical help, and to K. Golden and C. Stewart for their help in preparing the manuscript.

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Appendix B

Journal of Neuroscience Research 25:405–411 (1990)

Rapid Communication

Rescue of Basal Forebrain Cholinergic Neurons After Implantation of Genetically Modified Cells Producing Recombinant NGF

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Mouse 3T3 fibroblasts were genetically modified by transfection with a mammalian expression vector containing the rat β-nerve growth factor (NGF) gene. The transfected cell line, designated 3E, contains several hundred copies of the rat NGF gene and secretes high levels of biologically active NGF. Pieces of collagen gel containing the NGF-secreting 3E cells were grafted to the brains of unilaterally fimbria–fornix-lesioned rats. Grafts of the genetically modified NGF-producing cells rescued axotomized basal forebrain cholinergic neurons and significantly reduced cholinergic cell death in the medial septum as compared with rats treated with grafts of the parental 3T3 cells. Grafted fibroblast cells were detected, and rescue effects were noted up to 6 weeks after grafting. Local effects of NGF secreted by grafted cells were also seen at the gel–brain border in the form of sprouting acetylcholinesterase immunoreactive host cortical fibers. We suggest that implantation of genetically modified cells producing NGF may have therapeutic applications in rescuing damaged central cholinergic neurons in senile dementia of the Alzheimer type as well as in providing trophic support for chromaffin tissue grafts in Parkinson's disease.

Key words: hippocampus, fimbria-fornix, trophic factors

INTRODUCTION

Nerve growth factor (NGF) is known to influence sensory and sympathetic neurons in the periphery (reviewed by Levi-Montalcini, 1987) as well as central cholinergic neurons (Dreyfus, 1989; Whittemore and Seiger, 1987; Thoenen et al., 1987; Ebendal, 1989a). In this study, we have focused on the population of central cholinergic neurons in the basal forebrain that project, via the fimbria–fornix pathway, to the hippocampus. These neurons have NGF receptors (Richardson et al., 1986; Taniuchi et al., 1986; Hefti, 1986) and are thought to internalize NGF produced by pyramidal and granule neurons in the hippocampus (Ayer-LeLievre et al., 1988) and retrogradely transport it (Schwab et al., 1979) back to their cell bodies in medial septum. When the fimbria–fornix pathway is lesioned, the cholinergic neurons are thought to lose the trophic support of NGF obtained from the hippocampus, and, consequently, many of them degenerate and die. In support of such a trophic role, administration of exogenous NGF to fimbria–fornix-lesioned animals has been shown to prevent cholinergic cell death (Williams et al., 1986; Kromer, 1987) We show here that genetically modified cells, which continuously secrete high levels (approximately 5 ng/ml) of recombinant NGF (Ernfors et al., 1989a), can be grafted to the cavity after a fimbria–fornix lesion to provide an alternative chronic source of NGF in order to rescue axotomized neurons.

MATERIALS AND METHODS

In Situ Hybridization

Suspensions of 3T3 and 3E cells were smeared onto glass slides and air dried. The slides were fixed in paraformaldehyde (4%, 5 min), washed with PBS, dehydrated in ethanol, and treated with chloroform for 10 min. A 50-mer oligonucleotide antisense probe for rat NGF (sequence: 5′-CGC CTT GAC AAA GGT GTG AGT CGT GGT GCA GTA TGA GTT CCA GTG CTT GG-3′) was synthesized on a DNA synthesizer (Applied

Received October 9, 1989; accepted December 5, 1989.

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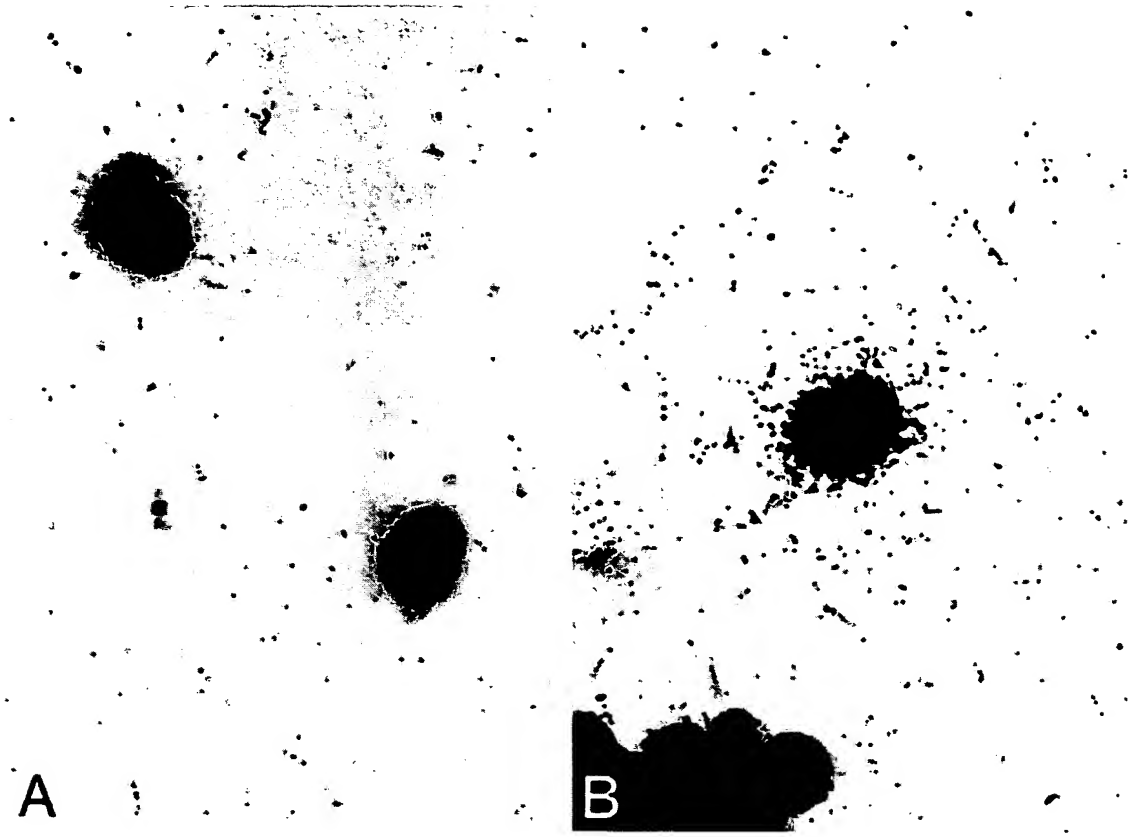

Fig. 1. NGF mRNA expression in the recombinant NGF-secreting 3E cells. Slides of paraformaldehyde-fixed cell smears of parental 3T3 (A) and 3E (B) cells were hybridized to an antisense oligonucleotide specific for NGF mRNA. Note the dense labeling overlying the cytoplasm of the 3E cells shown in B. Magnification A, B: ×510.

Biosystems 381A). Terminal deoxynucleotidyl transferase (IBI, New Haven, CT) was used to label the probe at the 3′ end with alpha-^{35}S-dATP (specific activity ~ 2 × 10^8 cpm/μg). Hybridizations were carried out as described previously (Ernfors et al., 1989b). Slides were washed in 0.1 × SSC (1 × SSC is 0.15 M NaCl, 0.015 sodium citrate, pH 7.0) + 0.1% sarcosyl at room temperature for 20 min, and in 0.1 × SSC at 54°C for 3 × 20 min, dried, dipped in photo emulsion (Kodak NTB-2, diluted 1:1 in 0.6 M CH_3COONH_4), and exposed for 7 weeks. Following development, cells were lightly counterstained with cresyl violet.

Lesion and Grafting Procedures

A unilateral aspiration lesion was made of the fimbria–fornix pathway in the brains of eight female Sprague-Dawley rats (ALAB Laboratorietjänst AB, Sollentuna, Sweden; 200–220 g body weight) under halothane anesthesia. A previously described stable mouse 3T3 cell line, designated 3E, in which several hundred copies of the rat NGF gene had been inserted and which expresses and secretes recombinant NGF (Ernfors et al., 1989a) was first grown alone in culture and then in a three-dimensional rat collagen gel matrix (Ebendal, 1989b). Similarly, the parent 3T3 cells were grown in parallel gels. The gels contained clusters of cells occupying approximately 5% of the gel volume prior to grafting. Immediately after the lesion was made, pieces of gel measuring approximately 25 mm^3 were inserted into the cavity formed by aspiration of a portion of the fimbria–fornix pathway and the gels covered by gel-foam. Four rats received grafts of pieces of collagen gel containing the transfected, NGF-producing cell line, and four received grafts of collagen gel containing the parent 3T3 cell line. As the 3T3 cells are of mouse origin, host rats were immunosuppressed with daily injections of cyclosporine A (10 mg/kg i.p.) and protected from infection by Vibramycin (2 mg/kg i.p., daily).

Immunohistochemistry

After 4–6 weeks rats were sacrificed under deep barbiturate anesthesia by transcardial perfusion of Ca^{2+}-

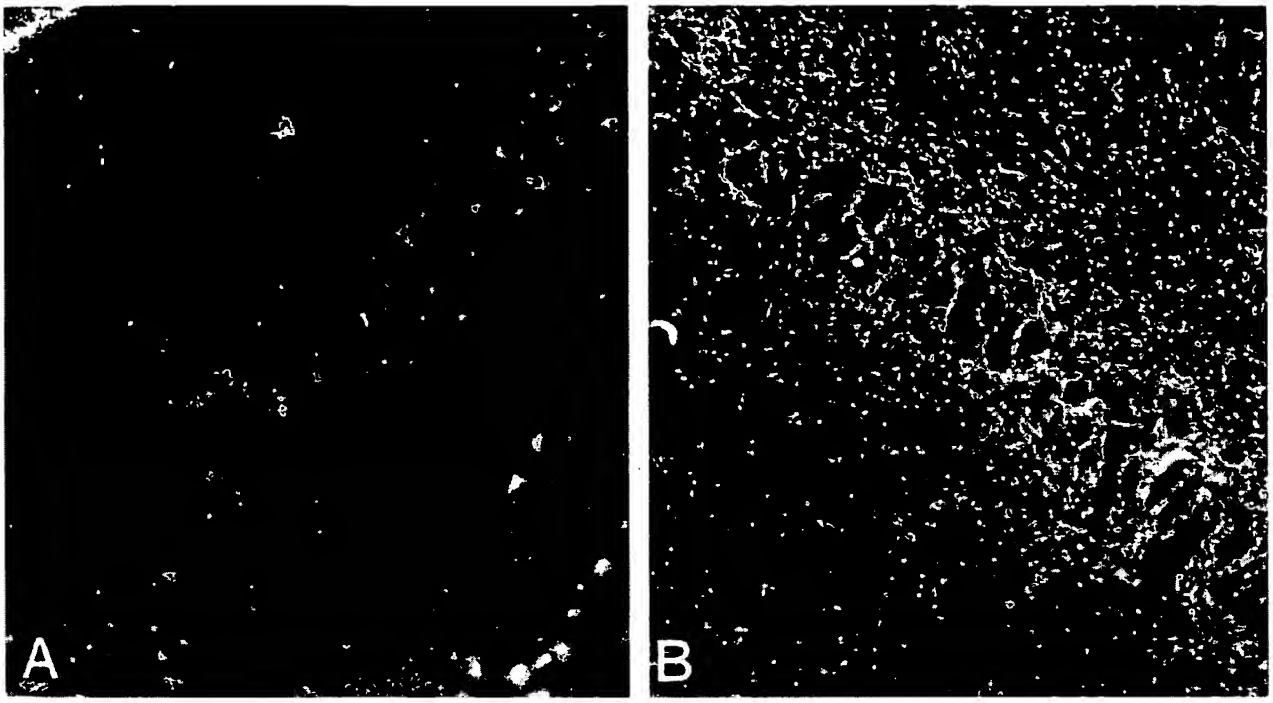

Fig. 2. Disappearance of cholinergic nerve terminals in the hippocampus following a fimbria–fornix lesion as revealed by AChE immunohistochemistry. Virtually all AChE-positive fibers have disappeared on the lesioned side (**A**), while the control side (**B**) is richly supplied with such fibers. Fluorescence microphotographs, magnification × 125.

free Tyrode's solution, followed by chilled 4% paraformaldehyde in phosphate buffer, and brains were processed for immunohistochemistry (Coons, 1958). Cryostat tissue sections (14 μm) were incubated with antibodies to acetylcholine esterase (AChE; generously provided by Drs. J. Grassi and J. Massoulie, France), followed by fluorescein-labeled swine anti-rabbit secondary antibodies. A comparison between AChE histochemistry, AChE immunohistochemistry, and choline acetyltransferase immunohistochemistry (Eriksdotter-Nilsson et al., 1988) suggests that AChE immunohistochemistry is a reliable marker of cholinergic neurons in the septum. As a further test, the distribution of AChE-immunoreactive neurons in the medial septum was compared with that of neurons immunoreactive with a monoclonal NGF-receptor (NGFR) antibody (MC192, kindly provided by Dr. Eugene Johnson, St. Louis, MO). All AChE-immunoreactive neurons in the medial septum–horizontal limb of the diagonal band of Broca region were counted on the lesioned as well as the unlesioned side in a series of approximately 20 sections taken from each brain. Completeness of the fimbria–fornix lesion was ascertained by examination of the fimbria–fornix area and, particularly, by the loss of AChE-immunoreactive fibers in the ipsilateral hippocampus.

RESULTS

Expression of NGF mRNA in 3E Cells Prior to Grafting

In situ hybridization using an oligonucleotide probe specific for rat NGF mRNA revealed a dense labeling overlying the cytoplasm of the majority of the 3E cells prior to grafting (Fig. 1A), indicating a high level of NGF mRNA expression, as previously suggested by Northern blot analysis of RNA prepared from these cells (Ernfors et al., 1989a). None or low labeling was seen over the parental 3T3 cells after hybridization to the same NGF-specific oligonucleotide probe (Fig. 1B). However, approximately 30% of the 3E cells were less intensely labeled, and a minority of cells (less than 10%) were apparently not labeled using the specific NGF probe.

Rescue of Axotomized Neurons With Grafted 3E Cells

The fimbria–fornix lesion caused complete or almost complete loss of AChE-immunoreactive nerve terminals in the hippocampus on the lesioned side without affecting the density of such fibers on the contralateral side (Fig. 2). Surviving fibroblasts were found in all gels

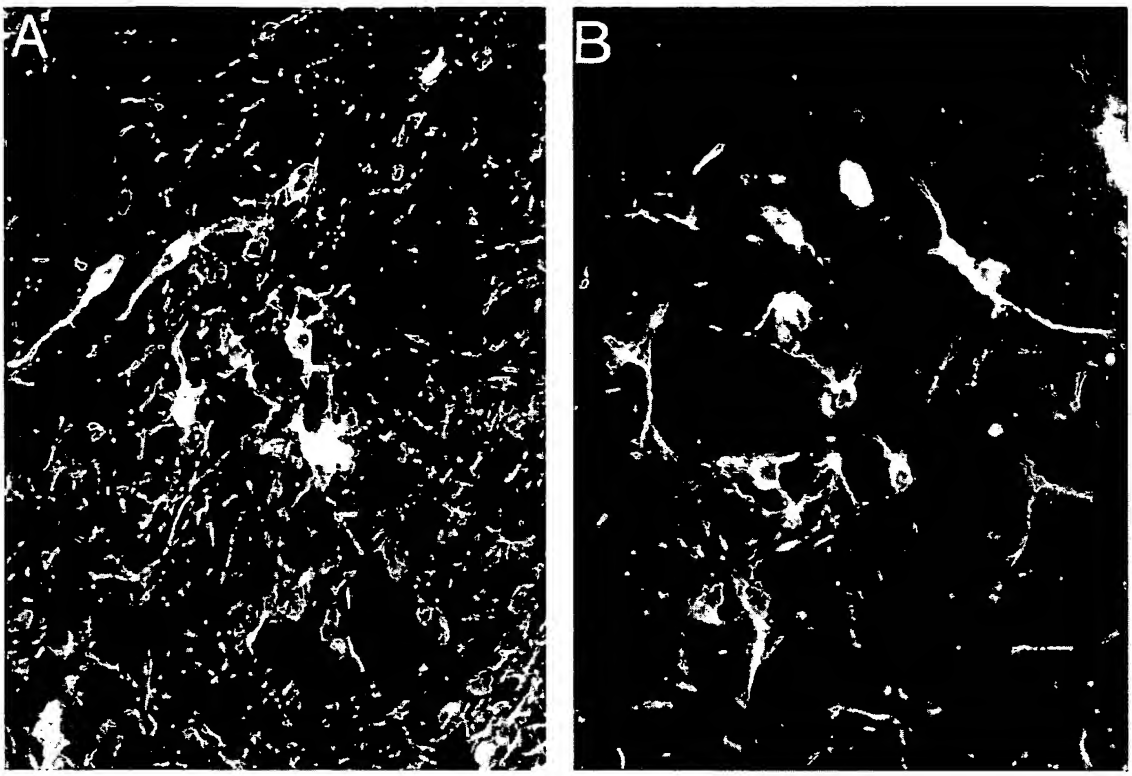

Fig. 3. Comparison of AChE-IR (**A**) and NGFR-IR (**B**) cells in the basal forebrain of normal rats. The two markers visualize a similar, if not identical, set of cells in the medial septum and basal forebrain. Note that more dendritic and axonal arborizations are immunoreactive with the AChE antibodies than with the NGFR antibody. Magnifications: A, ×235; B, ×225.

4–6 weeks after grafting, yet there was no indication that the cells had infiltrated the host brain tissue.

Basal forebrain cholinergic neurons and processes in septum and the diagonal band were strongly labeled with the AChE antibodies. The AChE-immunoreactive septal and diagonal band neurons (Fig. 3A) also showed strong NGF receptor-like immunoreactivity on their cell surface (Fig. 3B). The distribution of AChE- and NGFR-immunoreactive neurons in the diagonal band and medial septal area were found to be very similar, if not identical.

As illustrated in Fig. 4A, there was a clear loss of AChE-immunoreactive somata in the medial septum/vertical limb of the diagonal band area on the lesioned side in animals receiving grafts of 3T3 cell-containing gels. At the graft/host interface, AChE-IR nerve terminals from the surrounding cortex appeared to increase in density and sprout into the collagen gel in the presence of 3E cells (Fig. 4B). Little, if any, proliferative response was noted in host brain tissue adjacent to gels containing the parental cell line. The loss of AChE-immunoreactive cells was much smaller on the lesioned side in animals receiving gels containing NGF-producing 3E cells (Fig. 4C). The results of the cell counts, illustrated in Fig. 5, demonstrate that the percentage of AChE-immunoreactive cells lost in the medial septum on the lesioned side of the brain was significantly ($P = 0.0014$) reduced in the group of animals that received the NGF-producing 3E cells. Thus, grafts of gels containing NGF-producing 3E cells resulted in a significantly higher percentage of cholinergic neurons surviving axotomy, compared with animals treated with the parental cell line.

DISCUSSION

A wealth of recent information suggests that central cholinergic neurons depend on NGF for trophic support (for review, see Whittemore and Seiger, 1987; Thoenen et al., 1987; Dreyfus, 1989; Ebendal, 1989a). In particular, it has been shown that lesioned septal cholinergic neurons can be rescued by infusion or injection of exogenous NGF (Williams et al., 1986; Kromer, 1987). In order to obtain these beneficial effects, NGF must be available for an extended period of time and must be obtained from a local source in order to circumvent the blood–brain barrier. Repeated local injections or chronic infusion using osmotic minipumps connected to im-

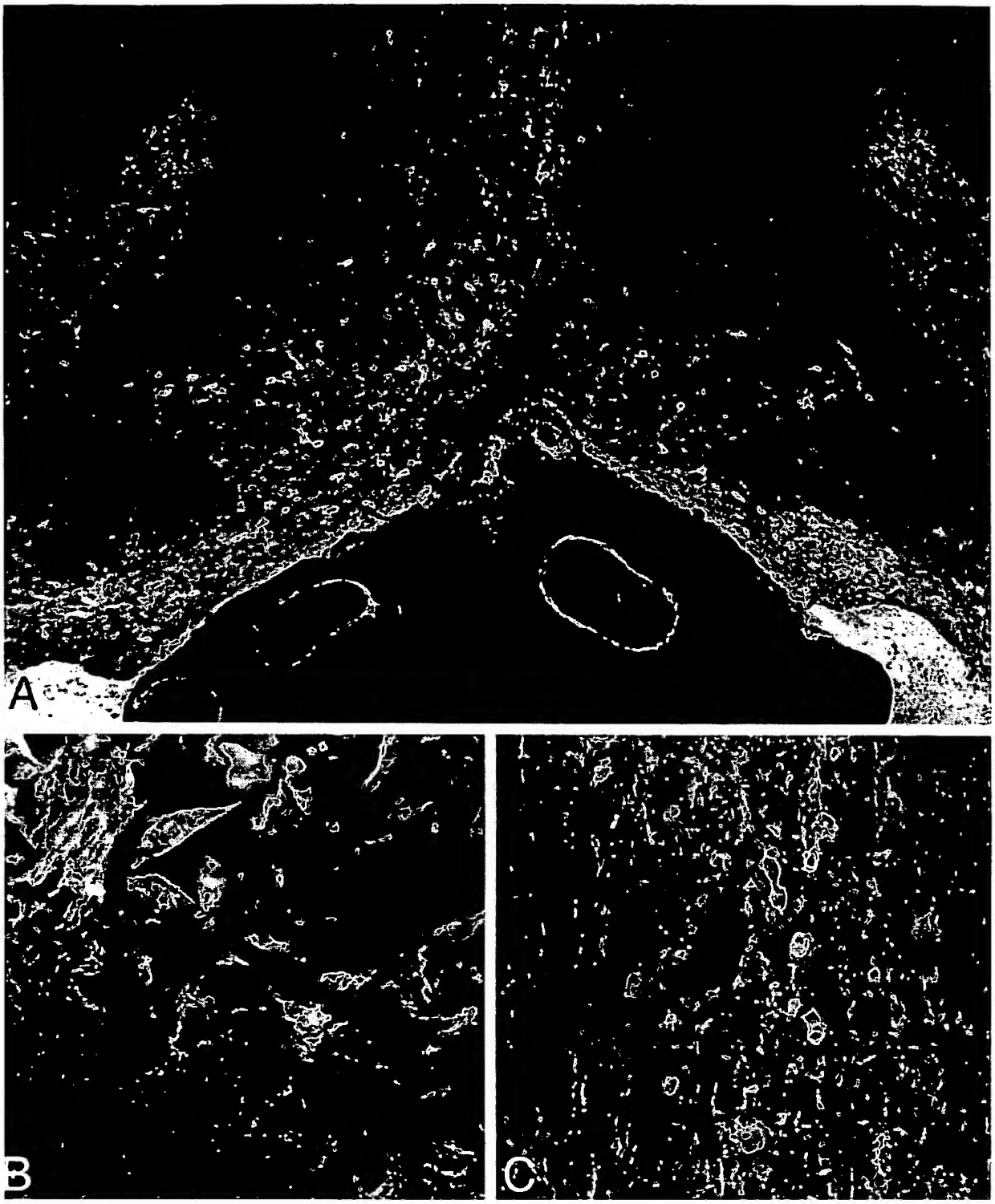

Fig. 4. Results of grafting collagen gels containing 3T3 (**A**) or 3E (**B and C**) cells to the fimbria–fornix lesion site. **A:** Relative loss of AChE-IR perikarya on the lesioned side (right) following grafting of a 3T3-containing gel, as compared with the intact control side (left). **B:** When NGF-secreting 3E cells are included in the gel, AChE-positive fibers from the surrounding cortical neuropil will sprout into the gel itself. Gel/host brain interface and gel, upper right; host brain, lower left. **C:** 3E-containing gels also rescued many more cells than 3T3 gels. Magnifications: A, ×70; B, ×210; C, ×315.

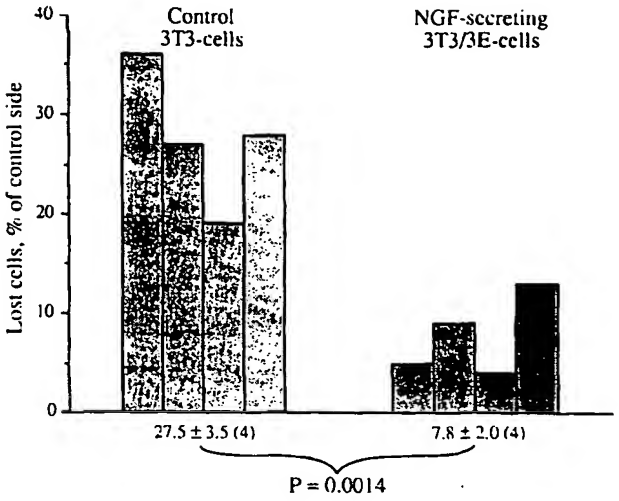

Fig. 5. Loss of basal forebrain cholinergic neurons 29–42 days after the fimbria–fornix lesion. Data obtained from eight animals, four receiving grafts of 3T3 cells, and four receiving grafts of 3E cells. Each bar represents the percentage of septal AChE-like immunoreactive cells lost on the lesioned side, as compared with the number of cells remaining on the unlesioned side.

planted cannulas or dialysis fibers (Strömberg et al., 1985) can provide NGF effectively for extended periods. However, these techniques require access to highly purified NGF.

Recently we (Olson et al., 1990; Ernfors et al., 1989a) and, independently, Rosenberg et al. (1988) have devised an alternative means of local NGF delivery, based upon grafting genetically modified cell lines that secrete biologically active recombinant NGF. These cells produce large amounts of NGF mRNA and secrete approximately 5 ng per ml of conditioned medium of biologically active recombinant rat NGF. However, in situ hybridization analysis of the 3E cells showed that the cells appear to comprise a heterogeneous population with regard to their level of NGF mRNA synthesis. Since the cells have been cloned twice by serial dilution and are consistently secreting NGF to their medium, it seems unlikely that the apparent heterogeneity is due to selection of more than one clone from the original transfection. Instead, NGF mRNA expression in the 3E cells may be cell cycle-dependent and, therefore, vary depending on where in the cycle the individual 3E cells are.

As described elsewhere (Ernfors et al., 1989a), genetically modified mouse 3T3 cells containing several hundred copies of the rat NGF gene can be grafted to the rat brain and can stimulate intrinsic cholinergic systems as well as support grafts of fetal cholinergic neurons. We demonstrate here that this NGF-producing cell line can also rescue axotomized cholinergic neurons of the medial septum–vertical limb of the diagonal band area, which would otherwise die following a fimbria–fornix lesion. These results support previous studies employing direct NGF administration, as well as a recent study (Rosenberg et al., 1988) using a retrovirus-transfected recombinant NGF-producing cell line in a similar experimental paradigm. The latter study reported cholinergic neuron survival for 2 weeks following grafting; we now report similar results up to 6 weeks postlesion, suggesting that the grafted cells can continue to produce NGF over extended periods of time and may provide a more "permanent" source of trophic support.

The loss of AChE-immunoreactive cells following grafting of the parent cell line was only some 35% in our hands, suggesting a partial rescue already effected by the control cell line. This is supported by the fact that the parent 3T3 cells do, indeed, contain low levels of NGF mRNA and secrete detectable amounts of biologically active NGF (Ernfors et al., 1989a). Using the transfected 3E cell line, however, which contains many-fold higher levels of NGF mRNA and which secretes 20–25-fold more NGF protein, almost all cholinergic neurons appeared to be present 4–6 weeks after injury.

We conclude that a genetically modified 3T3 fibroblast that secretes recombinant NGF can be used as a chronic local trophic support, rescuing damaged cholinergic neurons that would otherwise die. The fact that marked cholinergic deficits are found in Alzheimer's senile dementia (Bartus et al., 1982; Perry et al., 1978; Davies and Maloney, 1976) suggests that perhaps NGF and/or other growth factors might be of therapeutic value in this devastating disease. Similarly, experimental data (Strömberg et al., 1985) suggests that NGF might be used to support adrenal autografts in Parkinson's disease. It is, therefore, possible that genetically modified cells, secreting recombinant NGF or other growth factors, may be an effective way of providing long-term trophic support of neuronal populations in critical condition.

ACKNOWLEDGMENTS

This work was supported by the Swedish Medical Research Council (14X-03185, 12P-08868), Swedish Natural Science Research Council, Magnus Bergvalls Stiftelse, and U.S. Public Health Service grants NS 09199 and AG04418. C.W. was supported by the Life & Health Insurance Medical Research Fund. We thank Eva Lindqvist, Karin Lundströmer, Susie Marczinko, Monica Nyman, and Barbro Standwerth for technical assistance, and Annika Kylberg and Stine Söderström for help with preparing the fibroblast-containing collagen gels.

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